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Published in:
Journal of Cell Biology

DOI:
[10.1083/jcb.137.2.347](https://doi.org/10.1083/jcb.137.2.347)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1997

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

vanIjzendoorn, S. C. D., Zegers, M. M. P., Kok, J. W., & Hoekstra, D. (1997). Segregation of glucosylceramide and sphingomyelin occurs in the apical to basolateral transcytotic route in HepG2 cells. *Journal of Cell Biology*, 137(2), 347-357. <https://doi.org/10.1083/jcb.137.2.347>

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Segregation of Glucosylceramide and Sphingomyelin Occurs in the Apical to Basolateral Transcytotic Route in HepG2 Cells

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Abstract. HepG2 cells are highly differentiated hepatoma cells that have retained an apical, bile canalicular (BC) plasma membrane polarity. We investigated the dynamics of two BC-associated sphingolipids, glucosylceramide (GlcCer) and sphingomyelin (SM). For this, the cells were labeled with fluorescent acyl chain-labeled 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoic acid (C₆-NBD) derivatives of either GlcCer (C₆-NBD-GlcCer) or SM (C₆-NBD-SM). The pool of the fluorescent lipid analogues present in the basolateral plasma membrane domain was subsequently depleted and the apically located C₆-NBD-lipid was chased at 37°C. By using fluorescence microscopical analysis and a new assay that allows an accurate estimation of the fluorescent lipid pool in the apical membrane, qualitative and quantitative insight was obtained concerning kinetics, extent and (intra)cellular sites of the redistribution of apically located C₆-NBD-GlcCer and C₆-NBD-SM. It is demonstrated that both lipids display a preferential localization, C₆-NBD-GlcCer in the apical and C₆-NBD-SM in the basolateral area. Such a preference is expressed during transcytosis of both sphingolipids from the apical to the basolateral plasma membrane domain, a novel lipid trafficking

route in HepG2 cells. Whereas the vast majority of the apically derived C₆-NBD-SM was rapidly transcytosed to the basolateral surface, most of the apically internalized C₆-NBD-GlcCer was efficiently redirected to the BC. The redirection of C₆-NBD-GlcCer did not involve trafficking via the Golgi apparatus. Evidence is provided which suggests the involvement of vesicular compartments, located subjacent to the apical plasma membrane. Interestingly, the observed difference in preferential localization of C₆-NBD-GlcCer and C₆-NBD-SM was perturbed by treatment of the cells with dibutyryl cAMP, a stable cAMP analogue. While the preferential apical localization of C₆-NBD-GlcCer was amplified, dibutyryl cAMP-treatment caused apically retrieved C₆-NBD-SM to be processed via a similar pathway as that of C₆-NBD-GlcCer.

The data unambiguously demonstrate that segregation of GlcCer and SM occurs in the reverse transcytotic route, i.e., during apical to basolateral transport, which results in the preferential localization of GlcCer and SM in the apical and basolateral region of the cells, respectively. A role for non-Golgi-related, sub-apical vesicular compartments in the sorting of GlcCer and SM is proposed.

HEPG2 cells are well-polarized hepatoma cells which are regarded as a model system for hepatocytes (Chiu et al., 1990; Sormunen et al., 1993; Zaal et al., 1994). In polarized cells, the plasma membrane (PM)¹ is composed of two distinct domains, the basolateral and the apical membrane domain, of which the basolateral membranes form a continuous planar surface. HepG2 cells differ from other polarized cells because the apical membranes

are arranged into closed compartments located between two adjacent cells, thus representing the bile canalicular space (BC). Such apical BC membrane domains are believed to originate from intracellular microvilli-lined vesicles that mature under specific conditions of growth. It has been proposed that these vesicles are vectorially transported to the cell surface where they fuse with a similar, cell surface located microvilli-lined vesicle of an adjacent cell (Chiu et al., 1990).

Each membrane domain has its specific protein and lipid composition. To maintain such a unique composition, sorting mechanisms that regulate trafficking of molecules to the appropriate membrane domain must be operational. From studies on intracellular membrane traffic of proteins, a number of factors involved in the trafficking, sorting, and targeting of such molecules now emerges (Pimplikar and Simons, 1993; Mostov and Cardone, 1995). By contrast,

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1. *Abbreviations used in this paper:* BC, bile canalicular; GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; PM, plasma membrane; SM, sphingomyelin.

still little is known about lipid trafficking pathways and lipid sorting mechanisms.

Routes of lipid transport can be conveniently monitored by using fluorescently tagged lipids (Pagano and Sleight, 1985; Hoekstra and Kok, 1992). By this means we have previously shown that in HepG2 cells BC membranes are a target for lipids endocytosed from the basolateral surface (Zaal et al., 1993). Transcytosis of lipids has also been demonstrated in polarized Madin-Darby canine kidney (MDCK) cells (van Genderen and van Meer, 1995). However, whereas in these cells lipid transcytosis was shown to be bidirectional, in hepatic cells only basolateral to apical transcytosis has been demonstrated thus far. In addition to transcytosis, Golgi-derived de novo synthesized (glyco)-sphingolipids are transported to both the basolateral and apical domain in HepG2 (Zaal et al., 1993), MDCK (van Meer et al., 1987), and intestinal epithelial (Caco-2) cells (van 't Hof and van Meer, 1990). In the latter two cell lines it was demonstrated that newly synthesized glucosylceramide (GlcCer) and sphingomyelin (SM) were sorted for either the apical or basolateral PM domain. A sorting model was postulated in which the clustering of GlcCer in the luminal leaflet of the *trans*-Golgi network forms so-called microdomains. Subsequently, these microdomains, relatively poor in SM, bud off, thus giving rise to vesicles that are destined for the apical domain (van Meer and Simons, 1988). Current evidence suggests that also glycosylphosphatidylinositol (GPI)-anchored proteins can be part of these glycosphingolipid-enriched microdomains, and, moreover, that the preferential interaction of the GPI-anchor with these microdomains would account for their apical enrichment (Lisanti and Rodriguez-Boulant, 1990). Although direct, i.e., nontranscytotic, targeting of some Golgi-derived GPI-anchored proteins (Ali and Evans, 1990) as well as ceramide metabolites (Zaal et al., 1993) to BC has also been demonstrated in hepatocytes, specific sorting of GlcCer, and its subsequent targeting to BC has not been demonstrated in these cells.

In the present study, evidence is presented for the existence of a transcytotic route via which fluorescent acyl chain-labeled 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] hexanoic acid (C_6 -NBD) derivatives of GlcCer (C_6 -NBD-GlcCer) and SM (C_6 -NBD-SM) are transported from the apical BC to the basolateral PM. Apical to basolateral transcytosis has, to our knowledge, never been demonstrated before in hepatic cells. It is shown that in this reverse transcytotic route C_6 -NBD-GlcCer and C_6 -NBD-SM are sorted and subsequently flow to distinct PM domains, each lipid thus displaying a specific preference for localization. The experimental data indicate the involvement of vesicular compartments located subjacent to the BC, regulating trafficking and sorting in the apical to basolateral transport pathway.

Materials and Methods

D-sphingosine, sphingosylphosphorylsphingosine, 1- β -D-glucosylsphingosine, bovine serum albumin (BSA, fraction V) and monensin were obtained from Sigma Chemical Co. (St. Louis, MO). 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (C_6 -NBD) was from Molecular Probes (Eugene, OR). $Na_2S_2O_4$ (sodiumdithionite) was obtained from Merck (Darmstadt, Germany). Dibutyl cyclic AMP (db-cAMP) was purchased from Boehringer Mannheim (Mannheim, Germany). DMEM was

from GIBCO BRL (Life Technologies, Paisley, Scotland) and FCS was bought from BioWhittaker, Verviers, Belgium. All other chemicals were of analytical grade.

Cell Culture

HepG2 cells were grown in DMEM with 4.5 g of glucose per liter, supplemented with 10% FCS, and antibiotics. Media were changed every other day. For experiments, cells were plated in plastic culture flasks or, for microscopy, onto glass coverslips at low density ($\pm 20\%$ of surface occupied). In all experiments cells were used 3 d after plating. At this time the cells had reached an optimal ratio of polarity vs density, i.e., a maximal ratio of BC/cell.

Synthesis of C_6 -NBD-labeled Sphingolipids

C_6 -NBD-glucosylceramide, C_6 -NBD-sphingomyelin, and C_6 -NBD-ceramide were synthesized from C_6 -NBD and 1- β -D-glucosylsphingosine, sphingosylphosphorylcholine and D-sphingosine, respectively, as described elsewhere (Kishimoto, 1975; Babia et al., 1994). The lipids were purified by thin layer chromatography (TLC, see next section) until a single spot was obtained, cochromatographing with appropriate reference lipids (Babia et al., 1994). They were stored at -20°C and routinely rechecked for purity. The C_6 -NBD-lipids were quantified spectrophotometrically in an SLM fluorometer at excitation and emission wavelengths of 465 nm and 530 nm, respectively.

Incubation of Fluorescent Lipids with Cells

C_6 -NBD-Cer. Cells, grown on glass coverslips, were washed three times with a PBS solution. C_6 -NBD-Cer was dried under N_2 , redissolved in absolute ethanol and injected into Hank's balanced salt solution (HBSS, final concentration ethanol, 0.5% vol/vol) under vigorous vortexing. The cells were incubated with 4 μM C_6 -NBD-Cer under a CO_2 -containing, humidified atmosphere at 37°C for 30 min. Then, cells were washed three times with PBS to remove non-internalized probe, and further incubated at 37°C for 1 h. To allow microscopical examination of fluorescently labeled intracellular structures, the basolateral PM pool of fluorescent lipids was removed by incubating the cells in 5% (wt/vol) BSA-containing HBSS at 4°C for 2×30 min (back exchange). Subsequently, cells were washed three times with ice-cold HBSS and examined microscopically using an Olympus Provis AX70 microscope.

C_6 -NBD-GlcCer and C_6 -NBD-SM. Cells, grown on glass coverslips, were washed three times with PBS and incubated with 4 μM C_6 -NBD-GlcCer or C_6 -NBD-SM at 37°C (lipid dispersion was prepared as described above). After 30 min, the cells were washed with ice-cold PBS and the basolateral PM pool of C_6 -NBD-lipid was removed by back exchange at 4°C for 2×30 min, using 5% BSA-containing HBSS. Subsequently, cells were washed with ice-cold HBSS and examined microscopically. Occasionally, the cells were, subsequent to the back exchange and a PBS wash, rewarmed to 37°C and further incubated (chased) in HBSS at 37°C for various time intervals. In some experiments, the cells were chased at 37°C in HBSS, supplemented with 5% BSA. Note that during a 90-min incubation at 37°C in HBSS in the presence of BSA, only 0.7% of the total BSA fraction was taken up by the cells, as determined by measuring cellular uptake of rhodamine B isothiocyanate (RITC)-labeled BSA (which was added as a tracer to the BSA-medium; 0.5% of the nonlabeled BSA). Hence, these data exclude a potential interference of the cellular lipid pool with a lipid-analogue fraction that might have re-entered the cells after back exchange. Finally, artifacts in lipid flow and sorting resulting from treatment of the cells with BSA (fraction V) per se, were similarly excluded. Thus, identical results with respect to the fate of the lipid analogues, as described in this study, were obtained when the back exchange was carried out with either 15% FCS or nonlabeled dioleoylphosphatidylcholine vesicles (SUV, 500 nmol/ml) in HBSS.

After all final incubations, cells were washed with ice-cold HBSS and examined microscopically. For confocal laser scanning microscopy a TCS Leica (Heidelberg, Germany) apparatus equipped with an argon/crypton laser coupled to a Leitz DM IRB-inverted microscope was used. Images were converted to tagged-information-file format and printed on a Fujix P3000 printer.

To determine whether metabolic conversion of C_6 -NBD-GlcCer or C_6 -NBD-SM occurred during the incubations, cells, grown in culture flasks, were washed three times in PBS and incubated with 4 μM of either C_6 -NBD-lipid at 37°C for 30 min. After a subsequent back exchange procedure, cells were rewarmed and further incubated at 37°C for 90 min. After

gently scraping the cells with a rubber "policeman", cells and incubation media were extracted according to the method of Bligh and Dyer (1959). The lipids were analyzed by thin layer chromatography, using CHCl₃/methanol/NH₄OH/H₂O (35:15:2:0.5, vol/vol/vol/vol) as running solvent. Lipid amounts were quantified by scraping the spots from the TLC plates, followed by vigorous shaking in 1% (vol/vol) TX-100 in H₂O for 60 min. After removal of silica particles by centrifugation, NBD-fluorescence was measured as described above.

Quantification of Fluorescent BC Labeling with C₆-NBD-SM or C₆-NBD-GlcCer

Fluorescent BC labeling was quantified by the use of two different approaches: (1) Determination of the percentage NBD-positive BC and (2) calculation of BC-associated NBD-fluorescence. To determine the percentage of BC labeled with C₆-NBD-lipid, BC were first identified by phase contrast illumination, and then classified as being NBD-positive or NBD-negative under epifluorescence illumination using a filter set for blue excitation (BP 470-490/LP515). BC were only classified as being NBD-positive when labeled microvilli could be observed (cf. Figs. 1 and 2 B). Quantitative determination of BC-associated C₆-NBD-lipid was performed by using the capacity of sodiumdithionite to rapidly reduce NBD-fluorescence in the lumen and/or luminal leaflet of the BC (see Results). For this, cells were grown in plastic 25 cm² culture flasks and labeled with the fluorescent lipid analogues as described above. To determine the amount of BC-associated lipid, the cells were first subjected to a BSA/HBSS back exchange procedure, as described above, to remove the basolateral PM pool of C₆-NBD-lipid. Note that BSA has no access to the BC domain (see Results). By contrast, we observed that the irreversible NBD-quencher sodiumdithionite (McIntyre and Sleight, 1991) does acquire access to this membrane domain. Hence, after a BSA-back exchange, the cells were subsequently incubated with 30 mM sodiumdithionite in HBSS (diluted from a stock solution of 1 M dithionite in 1 M Tris-buffer, pH 10) at 4°C for 7 min. As a control, cells were incubated in HBSS in the absence of sodiumdithionite at 4°C for 7 min. After extensively washing (15 times with HBSS), cells were scraped, lipid was extracted, and fluorescence was measured as described above. The amount of C₆-NBD-lipid (expressed as pmol/mg protein) that was associated with BC was calculated from the following equation: $\text{NBD-fluorescence}_{\text{BC}} = \text{NBD-fluorescence}_{\text{control}} - \text{NBD-fluorescence}_{\text{dithionite-treated}}$.

Miscellaneous Procedures

Monensin Treatment. In the C₆-NBD-Cer-labeling experiments, cells were pretreated with 10 μM monensin at 37°C for 30 min. Monensin was present during the entire experiment. In the experiments in which transport of apically derived C₆-NBD-GlcCer or C₆-NBD-SM was investigated, monensin was added to the back exchange medium and was kept present in all following steps.

Db-cAMP Treatment. Cells were incubated with 4 μM C₆-NBD-SM or C₆-NBD-GlcCer in HBSS at 37°C for 30 min. After washing with ice-cold PBS, C₆-NBD-lipid residing in the basolateral PM was back exchanged. During the back exchange procedure 100 μM db-cAMP was included to preincubate the cells. After the second back exchange, cells were washed, rewarmed, and further incubated at 37°C in the presence of 100 μM db-cAMP.

Results

Basolateral to Apical Transcytosis and Quantification of Apically Located C₆-NBD-GlcCer and C₆-NBD-SM

To determine the intracellular fate of BC-associated C₆-NBD-GlcCer and C₆-NBD-SM, HepG2 cells were first incubated with 4 μM C₆-NBD-GlcCer or C₆-NBD-SM at 37°C for 30 min. After this time interval, both lipids were found intracellularly in vesicular structures (punctate fluorescence) and prominently labeled the BC and the basolateral PM (Fig. 1, *a-d*; *arrowheads*). The confocal micrographs, shown in Fig. 2, are entirely consistent with these observations and provide further support for the dominant localization of the lipid analogues in BC and basolateral

membranes. Note the typical "microvilli-like" appearance of fluorescence located in a BC formed between two adjacent cells (Fig. 2 *b*) when scanning through the plane of focus.

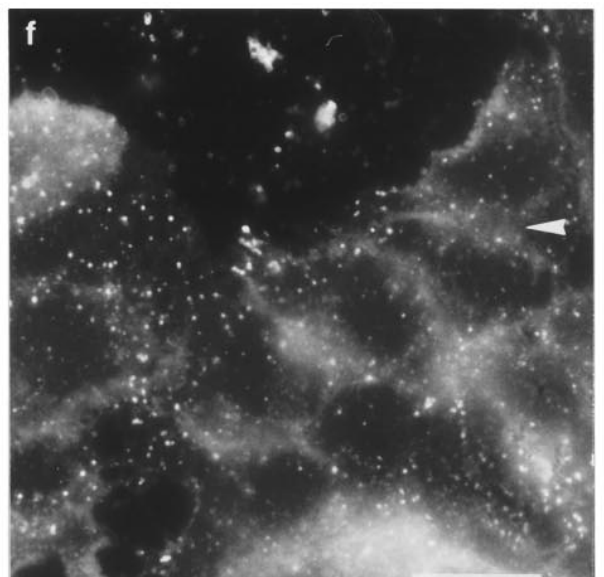
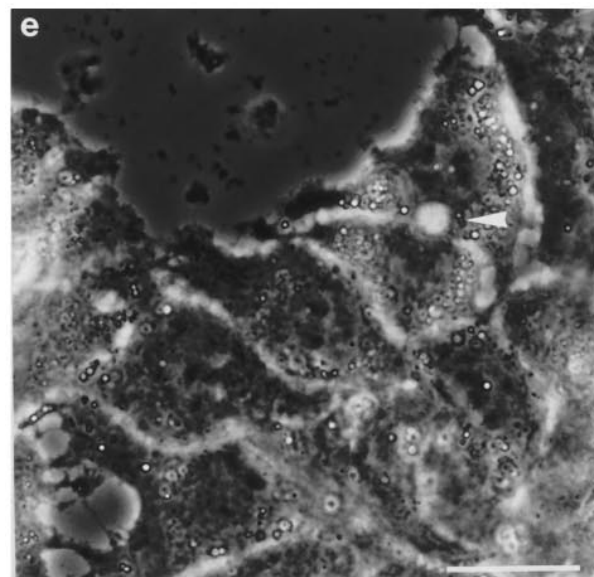
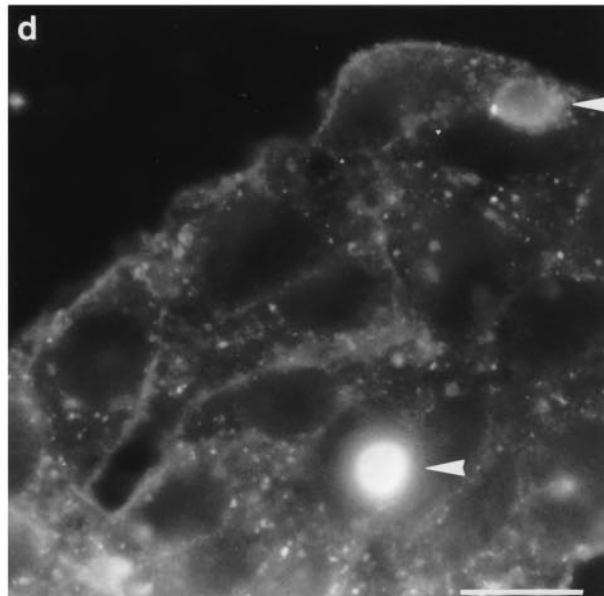
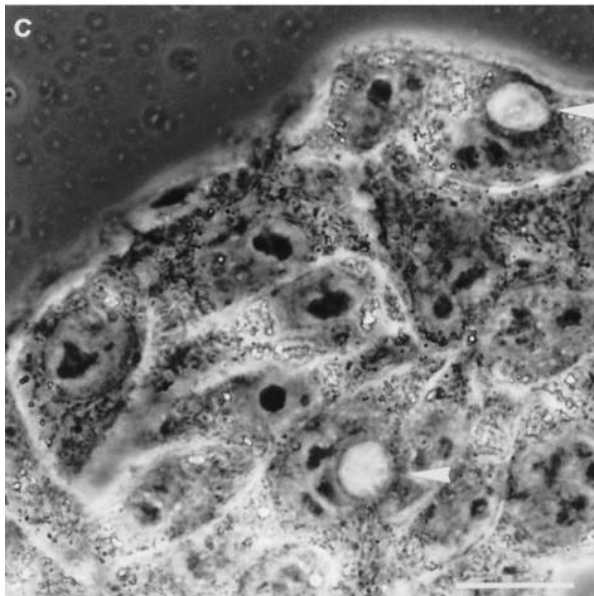
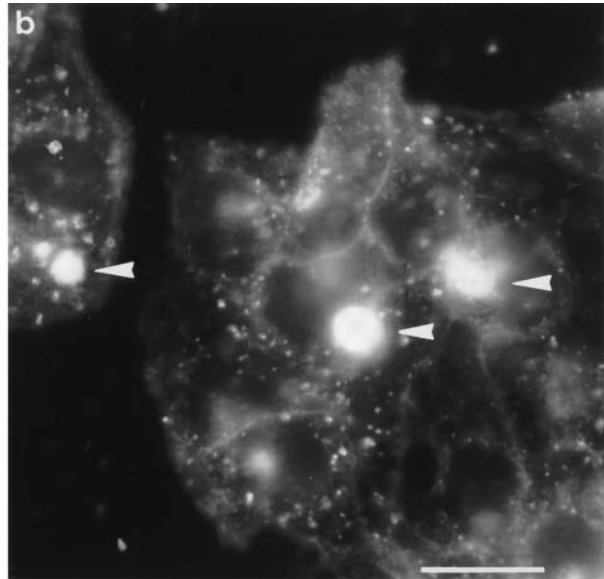
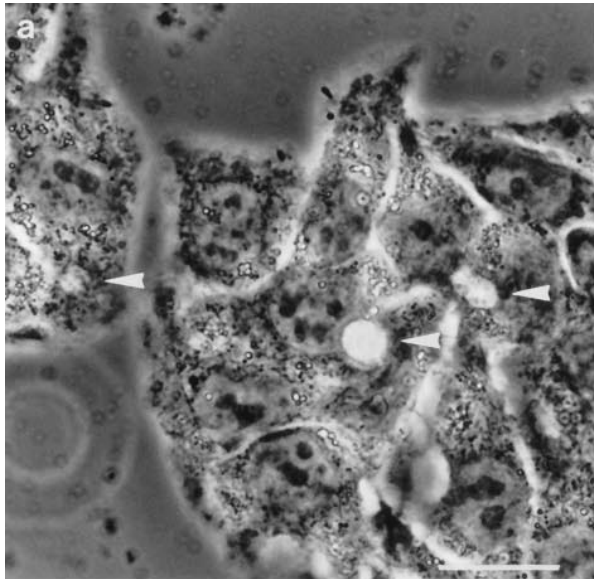
After the initial incubation at 37°C, C₆-NBD-GlcCer or C₆-NBD-SM residing in the outer leaflet of the basolateral PM was back exchanged by treatment of the cells with 5% BSA-containing HBSS at 4°C for 2 × 30 min. This procedure (see Materials and Methods section "C₆-NBD-GlcCer and C₆-NBD-SM") removed all NBD-fluorescence (>96%) from the basolateral PM, but not from the apical membrane (cf. Zaal et al., 1993). Using either lipid analogue, 70–75% of the BC, initially identified by phase contrast (see Materials and Methods), was scored as NBD-positive.

Because of the inaccessibility of C₆-NBD-lipids residing in the lumen and/or luminal leaflet of BC to the lipid scavenger BSA or acceptor vesicles, the quantification of BC-associated NBD-labeled lipid was considered as virtually impossible in this system. Recently, however, sodiumdithionite, which very slowly permeates across membranes, has been introduced as a useful agent to specifically and irreversibly quench fluorescent NBD-lipid analogues (McIntyre and Sleight, 1991; Pomorski et al., 1995). Here, we tested the potency of sodiumdithionite to quench C₆-NBD-GlcCer and C₆-NBD-SM that was located in the lumen and/or luminal leaflet of the BC. For this, cells were incubated with C₆-NBD-GlcCer or C₆-NBD-SM to label the BC, as described above. After a back exchange, the cells were incubated in HBSS supplemented with 30 mM sodiumdithionite at 4°C for 7 min. Subsequently, the cells were extensively (15 times) washed with ice-cold HBSS to remove the sodiumdithionite. Fluorescence microscopical analysis revealed a complete elimination of BC-associated fluorescence (Fig. 1, *e-h*; *arrowheads*). Presumably due to its slow membrane-permeating properties, sodiumdithionite did not significantly interfere with intracellularly located fluorescence, as reflected by an unchanged appearance of intracellular punctate fluorescence. However, these results do indicate that sodiumdithionite is apparently able to pass the tight junctions in HepG2 cells and, consequently, reaches the BC in a paracellular manner. Hence, by comparing NBD-fluorescence in cells before and after treatment with sodiumdithionite, the amount of BC-associated C₆-NBD-GlcCer and C₆-NBD-SM could be calculated (see Materials and Methods). We thus determined that in cells, labeled with C₆-NBD-GlcCer or C₆-NBD-SM as described above, 40.2 ± 5.0% of the total pool of cell-associated fluorescence was associated with BC (see below). This implies that sorting of either lipid during the early (30 min) transcytotic events does not take place, i.e., both lipids seem to follow the bulk flow in the transcytotic pathway.

After reaching the BC membrane, the question arises whether the observed lipid distribution reflects a steady state distribution or that a redistribution may occur, governed by a preferential residence of either lipid in the apical area. The next experiments were undertaken to address this issue.

Apically Derived C₆-NBD-GlcCer and C₆-NBD-SM Are Preferentially Located at Different Plasma Membrane Domains

After labeling the cells (30 min, 37°C) with either C₆-



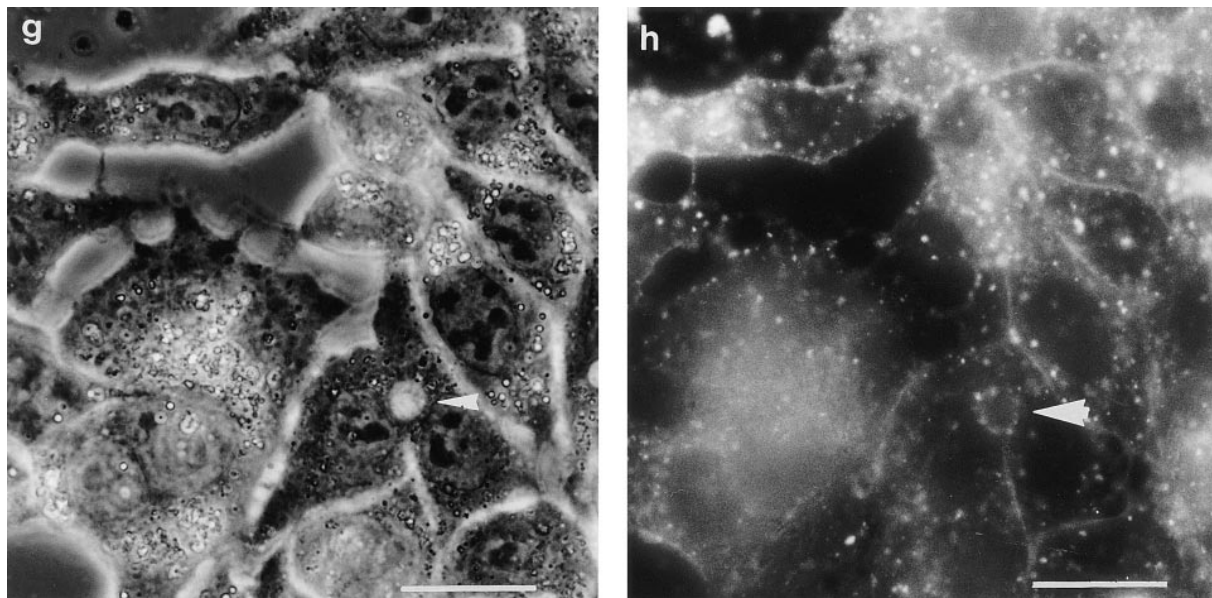


Figure 1. Labeling of HepG2 cells with exogenously administered C₆-NBD-GlcCer and C₆-NBD-SM. Cells were labeled with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C for 30 min. Both lipid analogues were present in the BC (arrowheads), the basolateral PM and intracellular vesicles (*b*, C₆-NBD-GlcCer; *d*, C₆-NBD-SM) (*a* and *c*, phase contrast to *b* and *d*). Note that in order to visualize intracellular fluorescence in *b* and *d*, basolateral membrane-located lipid analogues were partly removed by a brief back exchange procedure (5–10 min, 4°C). Alternatively, cells incubated as in *a* and *c* were subsequently subjected to a back exchange procedure at 4°C for 30 min to completely remove the basolateral PM pool of lipid analogues. After back exchange, the cells were incubated in HBSS, supplemented with 30 mM sodiumdithionite at 4°C for 7 min. After this incubation, the cells were washed 15 times with ice-cold HBSS to remove the sodiumdithionite. Note that after sodiumdithionite treatment C₆-NBD-GlcCer (*f*, phase contrast to *e*) nor C₆-NBD-SM (*h*, phase contrast to *g*) were detectable in the BC (arrowheads). Bar, 15 μ m.

NBD-GlcCer or C₆-NBD-SM and a subsequent depletion of the C₆-NBD-lipid from the basolateral PM, the cells were rewarmed to 37°C and incubated in HBSS for 30, 60, or 90 min. Over a 90-min incubation period, the percentage of BC labeled with C₆-NBD-GlcCer, as determined by fluorescence microscopy, remained nearly constant (Fig. 3, *hatched bars*). In cells labeled with C₆-NBD-SM (Fig. 3, *cross-hatched bars*), the percentage of fluorescently labeled BC progressively decreased from $71.5 \pm 8.8\%$ at time zero to $44.0 \pm 2.4\%$ after 60 min, after which the number of fluorescently labeled BC remained constant. However, although the percentage of C₆-NBD-SM-labeled BC, as detected by microscopical examination, did not further decline, the BC-associated fluorescence intensity did. As shown in Fig. 4, after a 60-min incubation period, the amount of BC-associated C₆-NBD-SM was hardly detectable anymore, as revealed by using the sodiumdithionite quenching assay. By contrast, and consistent with the microscopic observations, the amount of BC-associated C₆-NBD-GlcCer slightly decreased from 40 to $\sim 30\%$ of the total pool of cell-associated fluorescence (Fig. 4, *filled symbols*). Indeed, fluorescence microscopical analysis revealed that C₆-NBD-GlcCer remained mainly associated with BC and was also found in clusters of vesicles surrounding the BC (Fig. 5 *a*). Only a relatively faint labeling of the basolateral PM could be observed. After 60 min, C₆-NBD-SM was found to label mostly the basolateral PM while labeling of BC was hardly distinguishable (Fig. 5 *b*, compare to Fig. 1 *d*). Apparently, apically located C₆-NBD-GlcCer and C₆-NBD-SM were segregated for either

the apical or basolateral domains in HepG2 cells, respectively. Moreover, this segregation step appears to occur subsequent to basolateral to apical transport of the lipid analogues, i.e., after insertion of the lipid analogues in the apical membrane.

Segregation of C₆-NBD-GlcCer and C₆-NBD-SM Occurs during Apical to Basolateral Transcytosis

To clarify the extent to and the mechanisms by which C₆-NBD-GlcCer and C₆-NBD-SM were transported from the BC to the basolateral surface, the following experiment was carried out. After BC labeling with C₆-NBD-GlcCer or C₆-NBD-SM as described above, the C₆-NBD-lipid was chased at 37°C in HBSS, supplemented with 5% BSA to prevent re-internalization of the C₆-NBD-lipid once the analogue reached the basolateral membrane (see Materials and Methods). In a parallel experiment, the lipids were chased at 4°C. At 37°C, but not at 4°C (not shown), the percentage of BC labeled with C₆-NBD-GlcCer gradually decreased from $69.6 \pm 1.0\%$ at time zero to $68.3 \pm 2.0\%$, $45.0 \pm 4.9\%$, and $32.6 \pm 3.0\%$ after 30, 60, and 90 min, respectively (Fig. 6). At these conditions, i.e., in the presence of BSA, the absolute amount of BC-associated C₆-NBD-GlcCer was stable for 30 min after which it could no longer be detected as probed by the sodiumdithionite quenching assay (not shown). However, in cells labeled with C₆-NBD-SM, the percentage of NBD-positive BC decreased much faster, i.e., from $67.5 \pm 6.8\%$ at time zero to $42.0 \pm 3\%$ and $31.5 \pm 4.1\%$ after 30 and 60 min, respectively (Fig.

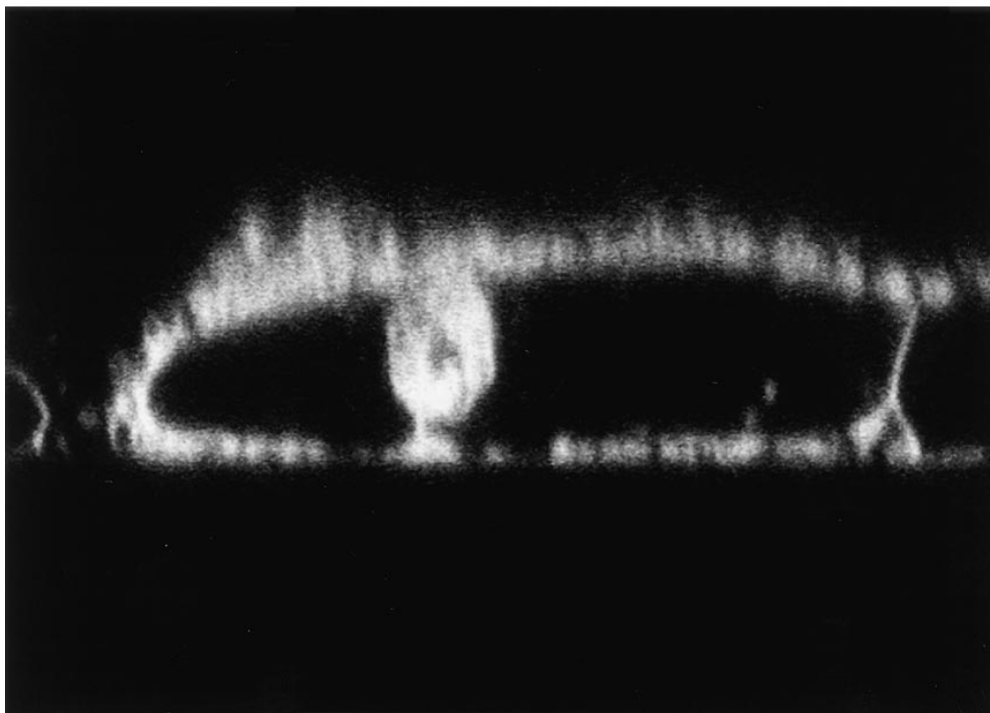
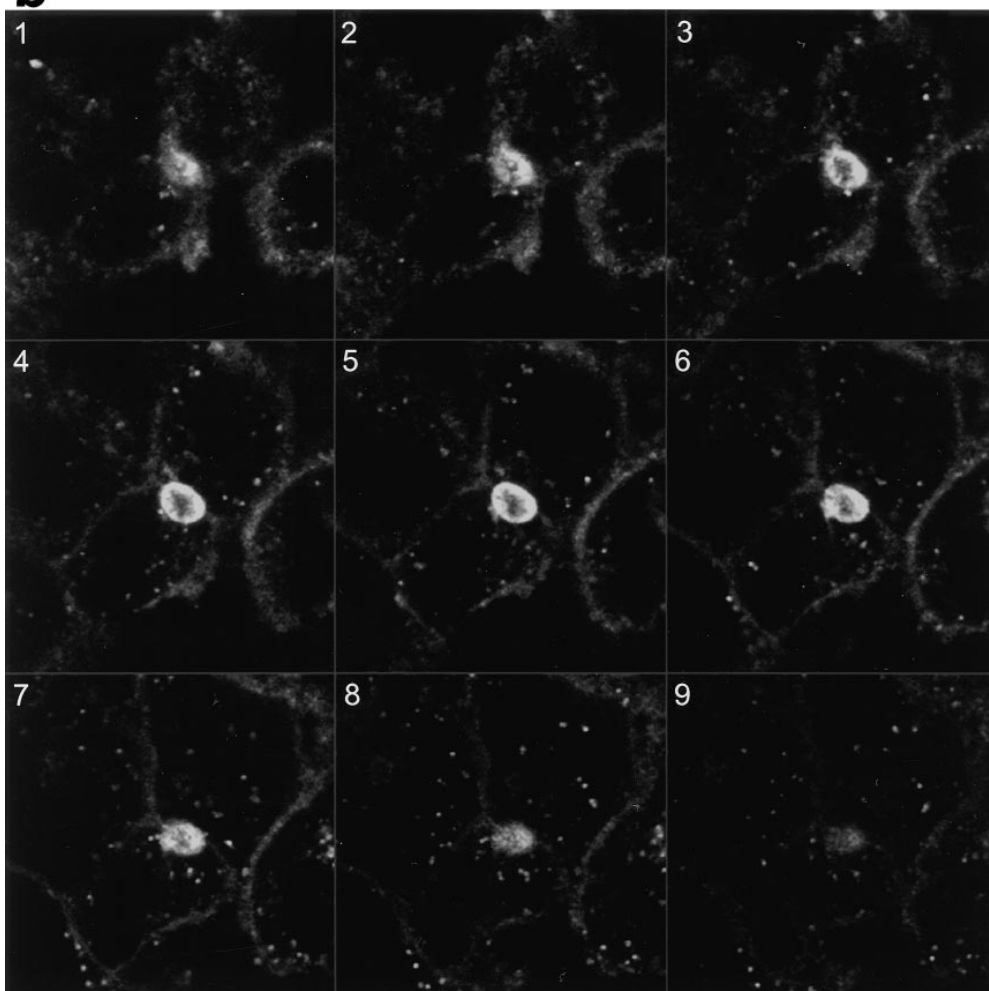
a**b**

Figure 2. Confocal laser scanning microscopy of C₆-NBD-GlcCer-labeled HepG2 cells. Cells, plated onto glass coverslips, were labeled with 4 μ M C₆-NBD-GlcCer at 37°C for 30 min. In *a*, a side view confocal image was taken with the plane of focus set in the center of the BC. In *b*, the cells were scanned from the top (1) to the bottom (9) of the BC. Prints were taken from optical sections of 0.5 μ m thickness.

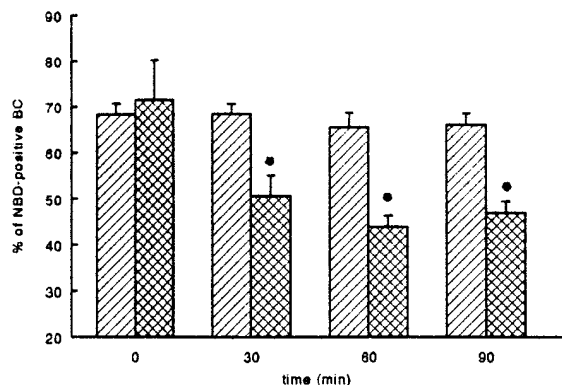


Figure 3. Disappearance of C₆-NBD-GlcCer and C₆-NBD-SM from BC. Cells were labeled with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C. After a 30-min labeling period, cells were washed and subjected to a back exchange procedure to remove the pool of fluorescent lipid associated with the basolateral PM. Then, cells were rewarmed to, and further incubated at 37°C for 0, 30, 60, or 90 min in HBSS. At each time point the percentage of NBD-positive BC in C₆-NBD-GlcCer-labeled cells (hatched bars) or C₆-NBD-SM-labeled cells (cross-hatched bars) (mean \pm SD of 4–6 experiments) was determined as described in Materials and Methods. * $P < 0.05$.

6). Prolonged incubation for up to 90 min did not result in a further decrease of the percentage of C₆-NBD-SM-positive BC, although BC-associated fluorescence intensities decreased in time. In fact, for both lipid analogues a nearly complete loss of the overall intracellular fluorescence was observed after 90 min (not shown). The loss of intracellular fluorescence could be entirely accounted for by the fluorescence recovered in the BSA-containing incubation medium, which is consistent with the notion that re-entry of BSA-complexed lipid is negligible (see Materials and Methods). Since these results were not observed at 4°C (data not shown), paracellular leakage of C₆-NBD-SM or C₆-NBD-GlcCer across the tight junctions could be excluded, and the data thus support a vesicular nature of lipid transport in the reverse transcytotic pathway.

During the time span of our experiments, only 10 and 7% of the total cell-associated pools of C₆-NBD-SM and C₆-NBD-GlcCer, respectively, were degraded after 2 h, with C₆-NBD-Cer as the main (i.e., >90%) metabolic product, as determined by lipid extraction and HPTLC analysis (see Materials and Methods). This implies that the observed redistribution of BC-associated C₆-NBD-SM or C₆-NBD-GlcCer was not due to conversion of the lipids. Based on these results we conclude that both C₆-NBD-GlcCer and C₆-NBD-SM can be retrieved from the apical domain after which they can be transcytosed to the basolateral surface. Note that the entire C₆-NBD-lipid content of at least 60–70% of the BC is involved in the reverse transcytotic pathway, as these fractions for both lipids can be efficiently removed in the presence of BSA (Fig. 3 vs Fig. 6).

Intracellular Sites Involved in the Segregation of C₆-NBD-GlcCer and C₆-NBD-SM in the Apical to Basolateral Transcytotic Route

Studies on apical endocytosis in various polarized cells have demonstrated that (a) apically internalized membrane

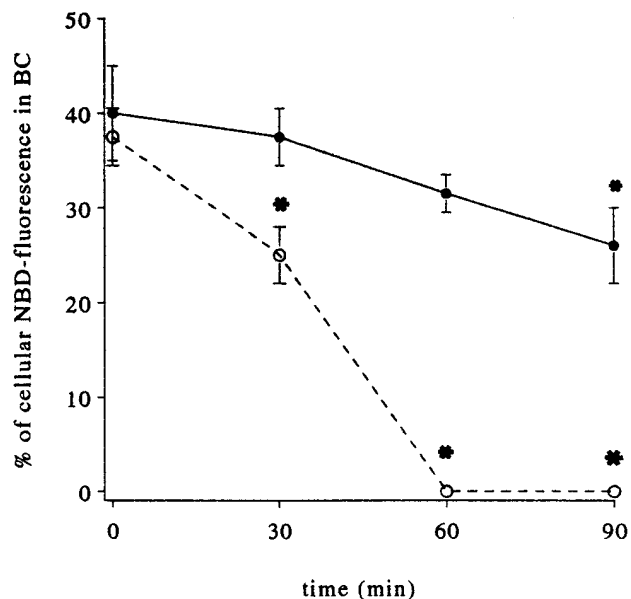


Figure 4. BC-associated C₆-NBD-GlcCer and C₆-NBD-SM after a chase in HBSS. HepG2 cells were grown in plastic 25 cm² culture flasks for 3 d. Cells were loaded with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C for 30 min. Then, C₆-NBD-lipid residing at the basolateral surface was depleted by BSA and cells were subsequently rewarmed to 37°C and further incubated in HBSS for 0, 30, 60, or 90 min. After another back exchange, the cells were incubated in HBSS (control) or HBSS supplemented with 30 mM sodiumdithionite (treated) at 4°C for 7 min. The amount of BC-associated C₆-NBD-GlcCer (filled symbols) or C₆-NBD-SM (open symbols) was then determined as described in Materials and Methods. Data are given as mean \pm SD of 4–6 experiments. * $P < 0.05$.

proteins can recycle from a tubulovesicular compartment, located subjacent to the apical PM (Hunziker et al., 1990; Apodaca et al., 1994; Barroso and Sztul, 1994) and (b) transport between sub-apical compartments and the apical PM can be affected by modulators of distinct signal transduction pathways (Barroso and Sztul, 1994; Cardone et al., 1994; Hansen and Casanova, 1994). cAMP and stimulators of protein kinase A (PKA) which are both members of the adenylate cyclase-cAMP-PKA signal transducing pathway, have been shown to positively modulate apically directed transport (Brignoni et al., 1995; Pimplikar and Simons, 1994). To investigate a possible role for cAMP in the segregation of C₆-NBD-GlcCer and C₆-NBD-SM in the reverse transcytotic pathway, cells were labeled with either lipid after which the basolateral PM pool of C₆-NBD-lipid was depleted by a back exchange as described above. During the back exchange procedure, which is carried out at 4°C, cells were preincubated with 100 μ M db-cAMP. After the back exchange, the cells were rewarmed and further incubated in HBSS in the presence of 100 μ M db-cAMP. As shown in Fig. 7, the percentage of NBD-positive BC in cells labeled with C₆-NBD-GlcCer remained constant for 90 min, as was the case in control cells (cf. Fig. 3). However, whereas the percentage of NBD-positive BC in cells labeled with C₆-NBD-SM rapidly decreased in control cells (cf. Fig. 3), this percentage of labeling remained virtually the same, for up to 90 min, in db-cAMP-treated cells (Fig. 7).

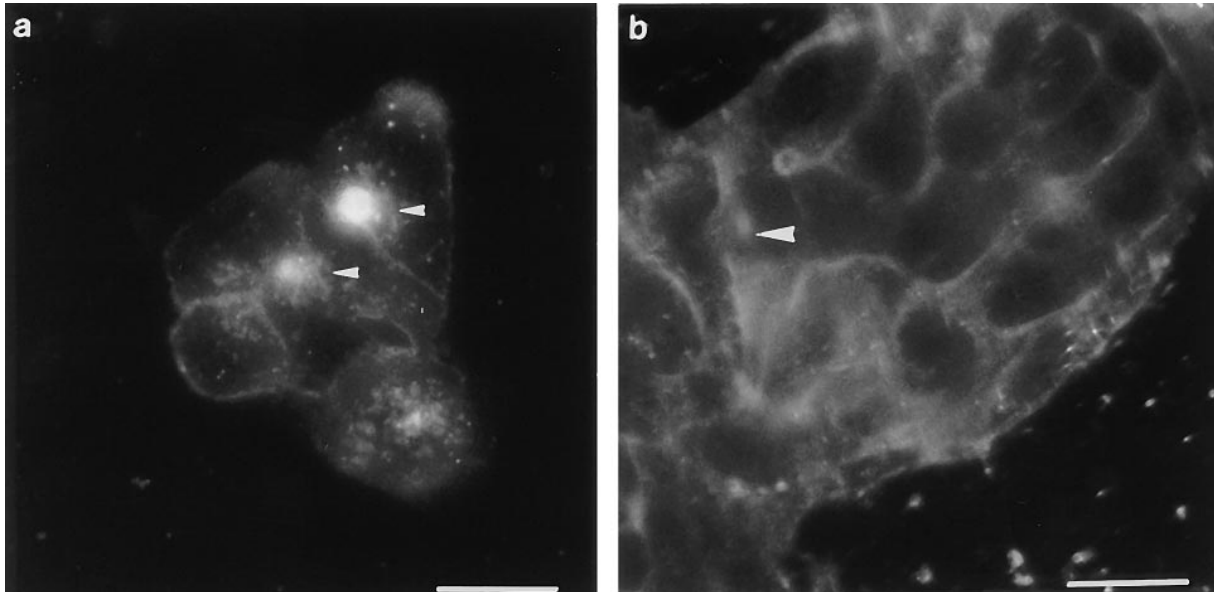


Figure 5. Trafficking of BC-derived C₆-NBD-GlcCer and C₆-NBD-SM. After the labeling of cells with C₆-NBD-GlcCer or C₆-NBD-SM as described in Materials and Methods, they were washed and subjected to a back exchange procedure, leaving only BC and some intracellular vesicles labeled. The cells were then rewarmed to, and further incubated at 37°C in HBSS (*a* and *b*). After incubation in HBSS for 60 min, C₆-NBD-GlcCer remained mainly associated with BC and clusters of vesicles surrounding BC (arrowhead), while only a faint labeling of the basolateral PM was observed (*a*). In contrast, C₆-NBD-SM mostly labeled the basolateral PM, whereas labeling of the BC was barely detectable (*b*; arrowhead). Bar, 15 μ m.

Although (fractions of) both lipids could still be transcytosed to the basolateral surface, as reflected by their back exchangeability after chasing BC-associated C₆-NBD-lipid in the presence of BSA, basolateral trafficking of both lipids was severely slowed down with regard to rate and extent (Fig. 8) in comparison to the basolateral flow in nontreated cells (cf. Fig. 6). Microscopic examination revealed

that in cells, not treated with dbcAMP, fluorescently labeled vesicles surrounding the BC were most prominently seen in cells labeled with C₆-NBD-GlcCer (Fig. 5). Interestingly, although essentially no BC-surrounding C₆-NBD-SM-labeled vesicles could be observed in nontreated cells (Fig. 5 *b*), such sub-apical NBD-fluorescent vesicles were

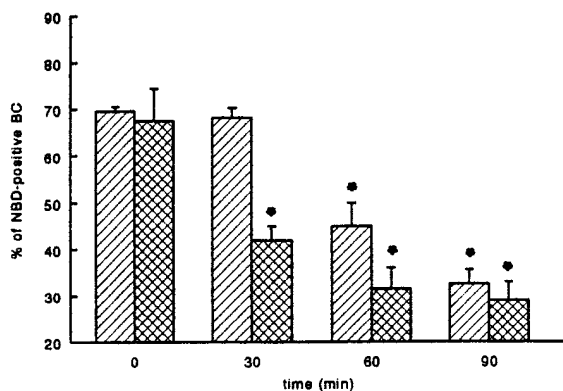


Figure 6. Disappearance of C₆-NBD-GlcCer and C₆-NBD-SM from BC after a chase in HBSS supplemented with BSA. Cells were labeled with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C. After a 30-min labeling period, cells were washed and subjected to a back exchange procedure to remove the pool of fluorescent lipid associated with the basolateral PM. Then, cells were rewarmed to, and further incubated at 37°C for 0, 30, 60, or 90 min in HBSS, supplemented with 5% BSA. At each time point the percentage of NBD-positive BC in C₆-NBD-GlcCer-labeled cells (hatched bars) or C₆-NBD-SM-labeled cells (cross-hatched bars) (mean \pm SD of 4–6 experiments) was determined as described in Materials and Methods. **P* < 0.05.

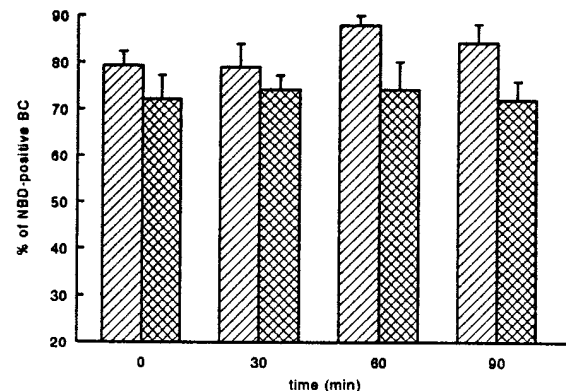


Figure 7. Effect of db-cAMP on the trafficking of C₆-NBD-GlcCer and C₆-NBD-SM from BC. Cells were labeled with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C for 30 min. After washing, the lipid analogues residing in the outer leaflet of the basolateral PM were depleted by subjecting the cells to a back exchange procedure. During this back exchange 100 μ M db-cAMP was added. Subsequently, cells were rewarmed to 37°C and further incubated in HBSS, supplemented with 100 μ M db-cAMP for various time intervals. At each time point, the percentage of NBD-positive BC was determined in C₆-NBD-GlcCer-labeled (hatched bars) and C₆-NBD-SM-labeled cells (cross-hatched bars) (mean \pm SD of 4–6 experiments), as described in Materials and Methods. **P* < 0.05.

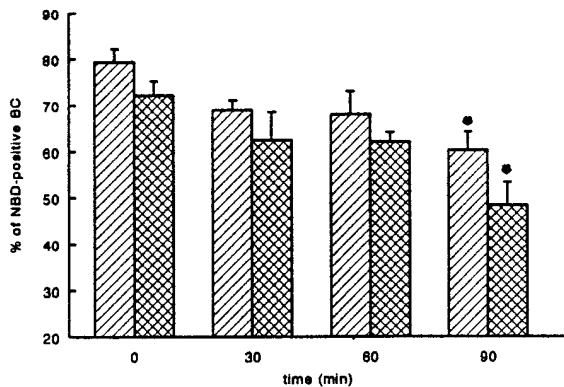


Figure 8. Effect of dbcAMP on trafficking of BC-derived C₆-NBD-GlcCer and C₆-NBD-SM in BSA-containing medium. Cells were labeled with 4 μ M C₆-NBD-GlcCer or C₆-NBD-SM at 37°C for 30 min. After washing, the lipid analogues residing in the outer leaflet of the basolateral PM were depleted by subjecting the cells to a back exchange procedure. During this back exchange 100 μ M db-cAMP was added. Subsequently, cells were rewarmed to 37°C and further incubated in HBSS, supplemented with 5% BSA, in the presence of 100 μ M db-cAMP for various time intervals. At each time point, the percentage of NBD-positive BC was determined in C₆-NBD-GlcCer-labeled (hatched bars) and C₆-NBD-SM-labeled cells (cross-hatched bars) (mean \pm SD of 4–6 experiments), as described in Materials and Methods. * $P < 0.05$.

readily distinguishable in db-cAMP-treated cells (Fig. 9). Hence, db-cAMP favored and/or promoted apically directed trafficking of both sphingolipids.

Sorting of C₆-NBD-GlcCer in a Sub-apical Compartment Does Not Involve the Golgi Complex

In hepatocytes, the Golgi apparatus is located near the bil-

iary pole. The organelle is well known as the main location for the assembly of biosynthetic products and secretory compounds (Farquhar, 1985). It has also been shown that endocytosed lipids can be transported to the Golgi apparatus (Schwarzmann and Sandhoff, 1990; Kok et al., 1991; Trinchera et al., 1991). Furthermore, after synthesis from their common precursor, C₆-NBD-Cer, C₆-NBD-SM, and C₆-NBD-GlcCer can be transported from the Golgi, where sorting is thought to occur, to both PM domains via direct, nontranscytotic routes in various polarized cell lines (van Meer et al., 1987; van 't Hof and van Meer, 1990; Zaal et al., 1993). To elucidate whether the sub-apical vesicular structures observed in our experiments might be Golgi-related and, thus, could be involved in governing the preferential localization of C₆-NBD-GlcCer in the apical area, we performed the following experiments. HepG2 cells were labeled with 4 μ M C₆-NBD-Cer at 4°C for 30 min, washed, and incubated at 37°C for 60 min in the presence of BSA. This resulted in an intense fluorescent labeling of the Golgi apparatus and the metabolic conversion of C₆-NBD-Cer into C₆-NBD-GlcCer and C₆-NBD-SM, as described previously (Zaal et al., 1993). C₆-NBD-GlcCer and C₆-NBD-SM, produced by the de novo synthesis, labeled approximately 66 \pm 4% of the BC. However, when the cells had been pretreated with monensin (10 μ M; Table I), only 17 \pm 3% of the BC was faintly labeled. Monensin is known to block vesicular transport from the Golgi apparatus to the PM and was shown to bring about a decrease in biliary lipid secretion (Casu and Camogliano, 1990). Since monensin treatment did not affect C₆-NBD-Cer metabolism (data not shown), our data show that monensin treatment of HepG2 cells, in agreement with results reported previously (Lipsky and Pagano, 1985; Kok and Hoekstra, 1994), inhibits (vesicular) lipid transport exiting from the Golgi. In the next experiment, cells were incubated with 4 μ M

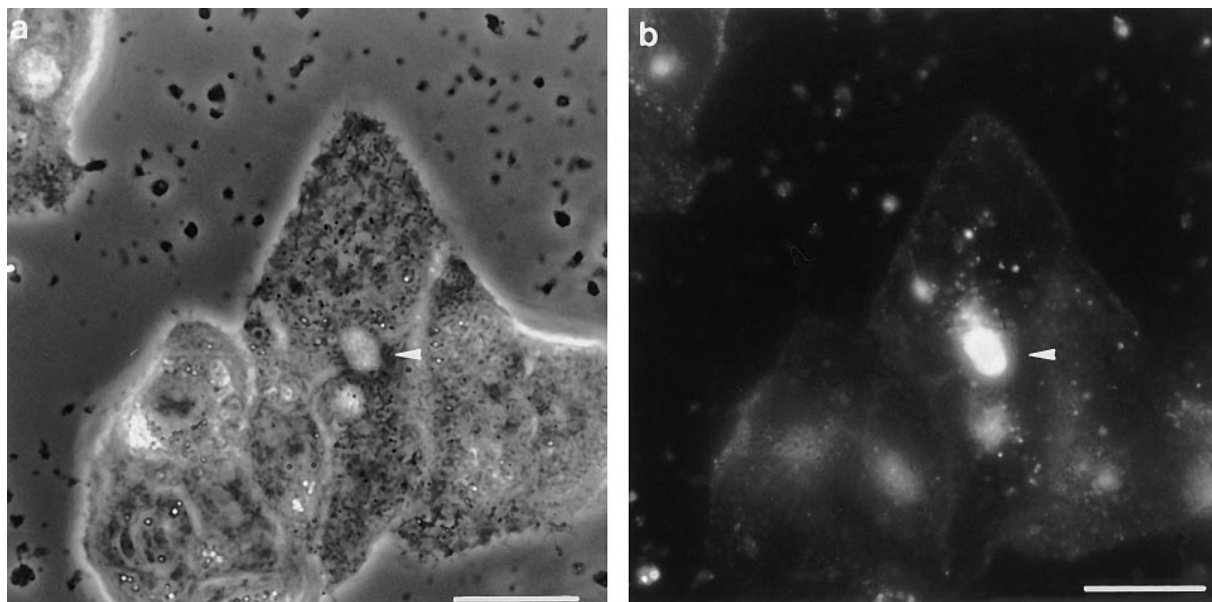


Figure 9. Effect of db-cAMP on the trafficking of BC-derived C₆-NBD-SM. Cells were labeled with 4 μ M C₆-NBD-SM at 37°C for 30 min, washed, and subjected to a back exchange procedure. During the back exchange 100 μ M db-cAMP was added. To monitor intracellular trafficking of the BC-derived lipid analogue, cells were rewarmed to 37°C and further incubated in HBSS, supplemented with 100 μ M db-cAMP for 60 min. C₆-NBD-SM labeled mostly BC and BC-surrounding vesicles (b; arrowhead) (a, phase contrast to b). Bar, 15 μ m.

Table I. Effect of Monensin on BC-labeling of Golgi-derived, De Novo Synthesized C₆-NBD-GlcCer and C₆-NBD-SM

| Percent of BC labeled with NBD-fluorescence | |
|---|---------|
| Control | 66 ± 4 |
| Monensin (10 μM) | 17 ± 3* |

HepG2 cells, grown on glass coverslips for 3 d, were pre-incubated in HBSS, supplemented with 10 μM monensin at 37°C for 30 min. The cells were labeled with 4 μM C₆-NBD-Cer at 37°C for 60 min, allowing internalization, metabolic conversion of C₆-NBD-Cer into C₆-NBD-GlcCer and C₆-NBD-SM, and subsequent transport of these metabolites. Subsequently, the cells were washed in HBSS and used for microscopical analysis. The percentage of NBD-positive BC (mean ± SD of five experiments) was determined as described in Materials and Methods. **P* < 0.0025.

C₆-NBD-GlcCer at 37°C for 30 min to label BC as described above. During the back exchange procedure 10 μM monensin was added to prevent transport of C₆-NBD-GlcCer from the Golgi to the BC in the subsequent steps. After this treatment, the cells were rewarmed to 37°C and incubated in the presence of 10 μM monensin and 5% BSA for 30 and 60 min. Compared to control cells, no difference in the percentage of labeled BC or BC-associated C₆-NBD-GlcCer was observed after the various incubation times (cf. Fig. 3). Similar results were obtained with cells that had been labeled with C₆-NBD-SM (data not shown). Hence, these results demonstrate that neither sorting of apically derived C₆-NBD-GlcCer nor apical to basolateral transport of C₆-NBD-SM involves the Golgi complex.

Discussion

In this study, we have presented evidence for the existence of a novel, vesicular lipid trafficking route by which (glyco)-sphingolipids are transcytosed from the apical, bile canalicular domain to the basolateral surface in HepG2 cells. Most importantly, in this apical to basolateral transcytotic route GlcCer and SM were segregated and preferentially targeted to the apical and basolateral PM domain, respectively. By contrast, sorting and a preferential transcytotic basolateral to apical transport of either GlcCer or SM was not observed after initial basolateral insertion. At those conditions, the lipids appear to follow bulk flow, similarly as observed for the flow of these sphingolipids in polarized MDCK cells (van Genderen and van Meer, 1995). The experimental data indicated that multiple sites were involved in governing the preferential apical localization of C₆-NBD-GlcCer, including the basolateral PM from which a rapid reflux of the lipid analogue could occur, which became apparent when monitoring its fate, following delivery to the apical membrane. The percentage of labeled BC, as well as the amount of BC-associated C₆-NBD-GlcCer, was more or less stable during a chase in HBSS (Fig. 3). Note that in the presence of BSA (Fig. 6) the percentage of C₆-NBD-GlcCer-labeled BC was, similarly, fairly constant after an initial chase of ~30 min. Subsequently, a rapid decline in the apical pool of C₆-NBD-GlcCer became apparent in the presence of the scavenger, but not in its absence. By contrast, irrespective of the presence of BSA, the patterns obtained for the percentages of C₆-NBD-SM-labeled BC were similar (Fig. 3 vs Fig. 6). Taken together, these data indicate that C₆-NBD-SM is rapidly transcytosed from the apical to the basolateral PM, whereas reverse

transcytosis of C₆-NBD-GlcCer is more gradual. Thus, the latter lipid displays a tendency to reside in and near the apical domain, whereas the fate of C₆-NBD-SM reflects a basolateral preference. The data also indicate that after a prolonged chase time (>30 min), a substantial fraction of the initial pool of apical C₆-NBD-GlcCer, may reach the basolateral membrane (Fig. 6). Evidently, as revealed by monitoring the chase in the absence of a scavenger, this pool apparently displays a rapid reflux to the apical membrane (Fig. 3).

In case of C₆-NBD-SM, the equilibrium that was established after a 60-min chase in HBSS did show a higher percentage of NBD-positive BC compared to the percentage seen after a chase in BSA-containing HBSS. This result indicates that also a reflux of basolateral arrived C₆-NBD-SM to BC occurred. The reflux of C₆-NBD-GlcCer, however, was much more extensive than that of C₆-NBD-SM. This suggests that the basolateral PM contributes to the distinct preferential localization of both lipids, consistent with the basolateral PM being a major site for sorting of apical membrane proteins in hepatocytes (Bartles et al., 1987; Schell et al., 1992). Yet, the delay in basolateral delivery of apical C₆-NBD-GlcCer in comparison to that of C₆-NBD-SM, as noted above, indicates that segregation of apically derived C₆-NBD-GlcCer and C₆-NBD-SM had occurred before these lipids reached the basolateral PM. Hence, the data suggest that multiple sites are involved in the distinct targeting of apically derived C₆-NBD-GlcCer and C₆-NBD-SM to their preferential domains.

Apically derived C₆-NBD-GlcCer appeared to be efficiently redirected to BC via non-Golgi-related sub-apically located vesicular compartments. Indeed, the inertness of sphingolipid trafficking of apically derived C₆-NBD-GlcCer and C₆-NBD-SM towards monensin treatment strongly supports the view that the Golgi apparatus was not involved in the intracellular processing of the apical pool of lipids. Furthermore, a "typical" Golgi fluorescence pattern, as reported previously for HT29 cells (Kok et al., 1991), was never observed. Although further work is needed, current evidence strongly suggests that the nature of the sub-apical compartment involved very likely relates to the compartments, described as part of the basolateral to apical transcytotic pathway (Hunziker et al., 1990; Apodaca et al., 1994; Barroso and Sztul, 1994; Hansen and Casanova, 1994). In fact, similar sub-apical tubulovesicular compartments, involved in transcytosis of IgA, have also been described in hepatocytes (Geuze et al., 1984; Hoppe et al., 1985). Moreover, also in hepatocytes, elevated concentrations of cAMP have been found to stimulate translocation of vesicles to the canalicular membrane (Boyer and Soroka, 1995). Indeed, dbcAMP promoted apically directed trafficking of both sphingolipids.

Given an apical enrichment, most C₆-NBD-GlcCer presumably underwent multiple rounds of recycling between the apical membranes and these sub-apical compartments. By contrast, C₆-NBD-SM was rapidly transcytosed from the BC to the basolateral surface and labeling of sub-apical compartments was never observed when C₆-NBD-SM was chased from BC (Fig. 5). Thus, transcytosis of C₆-NBD-SM might involve a pathway, entirely different from that of C₆-NBD-GlcCer, i.e., the analogue may bypass sub-apical compartments. However, treatment of the cells with db-

cAMP did reveal a sub-apical accumulation of C₆-NBD-SM, very reminiscent of that seen for C₆-NBD-GlcCer, in which C₆-NBD-SM accumulates before a redirection of trafficking to the apical membrane (Fig. 8). Hence, these observations would favor a scenario in which sub-apical compartments participate in the reverse transcytotic pathway of both lipid analogues. In such a compartment, C₆-NBD-GlcCer may be sorted in GlcCer-enriched microdomains which eventually bud off to be rerouted to the BC, whereas C₆-NBD-SM is rapidly transcytosed to the basolateral surface. Conditions where intracellular levels of cAMP are elevated (e.g., during biogenesis of apical [bile canicular] membranes) may then provide a signal for the targeting to and subsequent (apical) recycling of C₆-NBD-SM-labeled vesicles via sub-apical compartments. However, when the transcytotic flow of C₆-NBD-SM would entirely bypass that of C₆-NBD-GlcCer, the conclusion is still justified that sorting between both sphingolipids must occur, in this case at the level of the apical membrane. Hence either scenario, as depicted in this paragraph, clearly reveals the occurrence of sphingolipid sorting in the transcytotic pathway, a phenomenon which has not been shown before. The novel transport route as well as conditions that affect the flow through and sorting in this transcytotic pathway, as described in the present work, should thus pave the way to obtain clues as to the mechanisms of sphingolipid trafficking and sorting in polarized cells. Such work is currently in progress.

Received for publication 24 July 1996 and in revised form 27 January 1997.

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